Dihydropyridine-sensitive skeletal muscle Ca channels in polarized planar bilayers

2. Effects of phosphorylation by cAMP-dependent protein kinase

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ABSTRACT The effects of phosphorylation on the voltage-dependent properties of dihydropyridine-sensitive Ca channels of skeletal muscle were studied. Single channel currents were recorded upon incorporation of transverse tubule membranes into planar bilayers that were kept polarized at near physiological resting potential and subjected to depolarizing pulses under voltage clamp. Studies were conducted to analyze the properties of the channels at both the single channel and macroscopic level, using methods introduced in the preceeding paper (Ma et al., 1991. *Biophys. J.* 60: 890–901.). Addition of the catalytic subunit of cAMP-dependent protein kinase to the *cis* (intracellular) side of the bilayers containing channels resulted in: (*a*) an increase in open channel probability at all voltages above −50 mV; (*b*) a leftward shift (by 7 mV) in the curve describing the voltage-dependence of activation; (*c*) an approximate twofold decrease in the rate of inactivation; and (*d*) an increase in the availability of the channel. These findings provide new insights at the single channel level into the mechanism of modulation of the dihydropyridine-sensitive Ca channels of skeletal muscle by signal transduction events that involve elevation in cAMP and activation of the cAMP-dependent protein kinase.

INTRODUCTION

Ca channels in many cell types are regulated by receptor mediated events that involve activation of GTP-binding proteins and/or protein phosphorylation systems (for reviews see Hosey and Lazdunski, 1988; Brown and Birnbaumer, 1988). A problem in the study of these regulatory events is that channels from different sources have been preferred for electrophysiological vs. biochemical studies. Electrophysiologically, the Ca channels whose regulation has been studied most are the L-type channels of cardiac muscle (Tsien et al., 1986; Pelzer et al., 1990; Ochi and Kawashima, 1990). On the other hand, biochemical studies aimed at elucidating how such regulation might occur have used the DHP-sensitive channels from skeletal muscle (O'Callahan and Hosey, 1988; Jahn et al., 1988; Lai et al., 1990).

Calcium currents (I_{Ca}) of skeletal muscle are regulated by cAMP dependent events. I_{Ca} became larger and faster activating in the presence of β-adrenergic agonists (Arreola et al., 1987). The rate of ^{45}Ca influx into cultured cells increased when intracellular cAMP is elevated (Schmidt et al., 1985). Purified skeletal muscle calcium channels incorporated into liposomes were shown to be activated by either exposure to cAMP-dependent protein kinase (PKA) (Nunoki et al., 1989) or exposure of the muscle to cAMP activating agents prior to isolation (Mundiña-Weilenmann et al., 1991). Furthermore, the addition of PKA increased open probability of purified channels (Flockerzi et al., 1986) or native channels from T tubule vesicles (Yatani et al., 1988) incorporated into planar bilayers. However, the effect of phosphorylation by PKA on the voltage-dependent gating of skeletal muscle calcium channels has not been characterized well at the single channel level.

We undertook the present studies to improve the description of the functional effects of phosphorylation on skeletal muscle Ca channels. We used the techniques developed in the first paper of this series (Ma et al., 1991) to determine the effects of cAMP-dependent protein kinase on the voltage-dependent properties of the channels incorporated in planar lipid bilayers. The studies resulted in a description of the effects at both the single channel and macroscopic levels.

METHODS

Single channel recording and analysis of data

Preparation of transverse tubule membranes, their fusion with lipid bilayers and single channel recording were carried out as described in the previous paper (Ma et al. 1991). The membranes were kept polarized at −80 mV and depolarizing pulses elicited channel opening, manifested by inward Ba^{2+} currents. As in the previous paper, all the analyses were carried out on test-minus-control current records. The

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analysis included studies on single channel records and on their averages over large numbers of sweeps. At the single channel level, amplitude and open time histograms were constructed. The average open probability ($\bar{P}_0$) was calculated as fractional open time during the whole pulse. As discussed in the previous paper, $\bar{P}_0$ is an average over time of the nonstationary probability $P_0(t, V)$. Its voltage dependence can be described by a Boltzmann function,

$$\bar{P}_0 = \bar{P}_{max} \frac{1}{1 + e^{-(V - \bar{V})/K}}. \quad (1)$$

At the macroscopic level, the ensemble averaged currents $\langle I(t, V)\rangle^*$ were fitted by the function

$$I_{max}[1 - \exp(-t/\tau_m)]^3 \exp(-t/\tau_h), \quad (2)$$

where $I_{max} \equiv A\, m^3(\infty)$, A is a constant, and $m(\infty)$, τ_m and τ_h are functions of pulse voltage.

Protein kinase studies

The catalytic (C) subunit of cAMP-dependent protein kinase (PKA) was purified to homogeneity from bovine heart according to Sugden et al., (1976). It was introduced in the *cis* chamber (volume = 3 ml) to a final concentration of 0.25 to 1 μM, in the presence of 3 mM ATP-Mg. The chamber compartment was stirred for 1 min and the measurements of channel activity in the presence of PKA were started two to three min after introduction of the enzyme. The conditions used here for the phosphorylation experiments was very similar to those of previous experiments in which phosphorylation of the channel proteins in T-tubule membranes was directly studied with [γ-^{32}P]ATP. Under these conditions, the addition of PKA led to the phosphorylation of the α_1 and β subunits of the channel protein, with approximately five-fold more phosphate incorporated into the α_1 than the β subunit (O'Callahan et al., 1988; O'Callahan and Hosey, 1988).

Statistics

The voltage dependence of channel open probability and the time dependence of averaged currents were fitted with theoretical functions using nonlinear least-squares routines (Scarborough, 1966). In these cases a standard error of the parameters (σ) was calculated as the square root of the diagonal elements of the covariance matrix (Cleland, 1977). A 95% confidence interval for parameters determined in this manner includes ± 3 σ instead of ± 2 σ, owing to the nonlinear nature of the problem (Hamilton, 1969).

RESULTS

Routinely, after a channel was incorporated successfully in a bilayer, a sequence of several pulses to different voltages was applied in the reference solutions. Then PKA was introduced in the *cis* compartment and the same sequence of pulses was repeated. In some cases the measurements were started directly in the presence of PKA.

Representative ionic currents in reference conditions and after the addition of PKA are shown in Fig. 1. Several effects of PKA are illustrated by the records: (*a*) the open channel probability increased at all voltages; (*b*) the voltage dependence seemed to shift to lower voltages since open channel events appeared at −50 mV after the addition of PKA but not under reference conditions; (*c*) the gating kinetics changed; specifically, open events late in the pulse became much more frequent, and (*d*) the single channel conductance did not change. All these observations are analyzed in detail below. Data will be presented first on microscopic properties of the single channel, and then on macroscopic properties of the ensemble averaged currents.

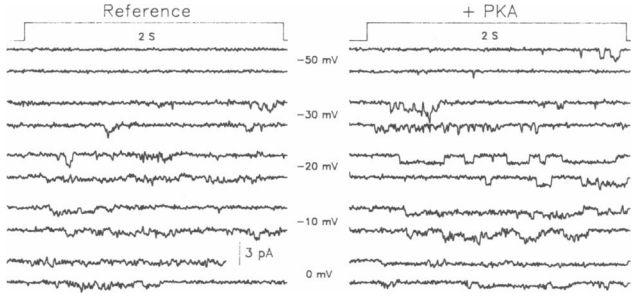

FIGURE 1 Phosphorylation by PKA increased the voltage dependent opening of the Ca channel. Selected single channel records at different membrane potentials (listed in mV) taken from one bilayer experiment in reference solutions (*A*) and a different experiment, 2–7 min after addition of 0.25 μM purified C-subunit of PKA to the *cis* chamber.

Voltage-dependent activation

The time-averaged open probability $\bar{P}_o$ vs. pulse voltage V in reference (*open circles*) and after introduction of PKA in the *cis* compartment (*solid circles*) is depicted in Fig. 2. Each data point in PKA is an average of six separate experiments. The vertical bars span ± one standard error of the mean. The solid curves represent the best fit Boltzmann functions of voltage (Eq. 1). The addition of PKA increased $\bar{P}_{max}$ from 0.078 (SD = 0.01) in the nonphosphorylated condition to 0.12 (0.01) in PKA (a significant difference, $p < 0.05$). In addition, $\bar{V}$ changed from −26 (0.2) mV to −33 (0.4) mV, confirming the suggestion from Fig. 1 that phosphorylation shifted the voltage dependence of channel activation so that the open events appeared at lower voltages. This 7 mV difference, though small, was significant (using ± 3 σ as 95% confidence intervals for the estimates of $\bar{V}$, cf Methods). The dotted curve in Fig. 2 represents the best fit Boltzmann in reference, scaled to match the final level in PKA, and demonstrates graphically the significance of the shift. K did not change (7.0 [0.14] mV in reference, 7.3 [0.38] mV in PKA).

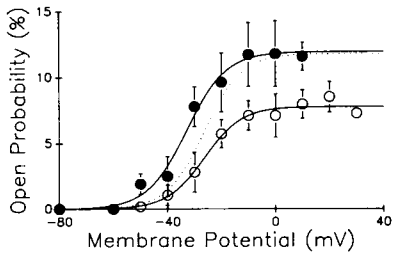

FIGURE 2 Effect of PKA on the voltage dependence of the open probability. Each data point represents the average of six separate bilayer experiments performed in the presence of 0.25 μM C-subunit of PKA in the *cis* chamber (*solid circles*). The open circles represent average values in 10 bilayers in reference condition. The curves represent Boltzmann functions (Eq. 1). The best fit parameters after PKA were: $\bar{P}_{max} = 0.12$ (SD = 0.01), $K = 7.3$ (0.4) mV, $\bar{V} = -32.6$ (0.4) mV. The parameter values in reference (Ma et al. 1991) were: $\bar{P}_{max} = 0.078$ (0.01), $\bar{V} = -26$ (0.2) mV and $K = 7.0$ (0.2) mV. The dotted curve is the best fit Boltzmann to the reference results, scaled to match the $\bar{P}_{max}$ after PKA.

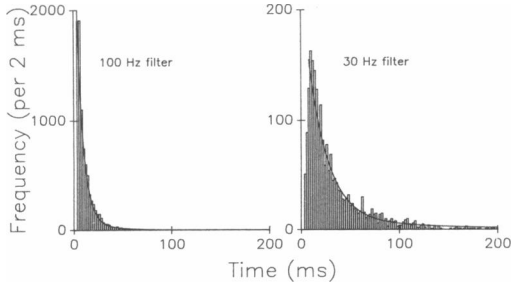

FIGURE 3 Open time distribution after exposure to PKA. Channel open times at −10 mV were computed from 192 episodes. At 100 Hz filter frequency (*left*), a total of 8,687 open events were detected. When the same calculation was carried out after a step of digital filtering at 30 Hz (*right*), the number of open events detected was reduced to 2,206. Both histograms were best fitted by a sum of two exponential distributions (Eq. 2). The best fit values of the parameters at 100 Hz were: $W_1 = 20{,}195$, $\tau_1 = 6.5$ ms, $W_2 = 2{,}929$, $\tau_2 = 22.1$ ms. At 30 Hz: $W_2 = 4{,}290$, $\tau_2 = 19.8$ ms, $W_3 = 1{,}114$, $\tau_3 = 101$ ms.

Single channel conductance and distribution of open times

Amplitude histograms were constructed from 32 test-minus-control sweeps, of 2 s duration, obtained after exposure to PKA. As in the absence of the protein kinase (Ma et al., 1991, Fig. 4) the histograms were well fitted by a sum of two gaussians. The central value of the one describing open channel events was at 0.49 and 0.70 pA, respectively at test voltages of 0 and −20 mV. The corresponding slope conductance, 10 pS, was not significantly different from the reference value of 9 pS. Thus, the addition of PKA did not alter measurably the open channel conductance.

Defining the threshold for current detection, φ, at 0.3 pA, a histogram of open times was constructed from the records at −10 mV, and is shown in Fig. 3 *A*. As in reference, the sum of two exponentials provided a good description. The best fit time constants were $\tau_1 = 6.5$ ms and $\tau_2 = 22$ ms. These numbers are very similar to those of the reference channel ($\tau_1 = 5.8$ and $\tau_2 = 30$ ms; Ma et al., 1991). The proportion W_2/W_1, of long over short events changed slightly, from 0.13 in reference to 0.15 in PKA.

It was shown in the previous paper that heavier filtering of the records in reference led to the appearance of a group of long openings, with a time constant (τ_3) of 92 ms. We repeated the procedure after the addition of PKA. Fig. 3 *B* shows the open time histogram obtained from the same records (Fig. 3 *A*) after digitally filtering at 30 Hz. As was found in reference, the histogram could be fitted after the initial 10 ms by a sum of two exponentials, with parameters $W_2 = 4290$, $\tau_2 = 20$ ms, $W_3 = 1110$, $\tau_3 = 100$ ms. The time constants were not different than in reference; the ratio W_3/W_2 was 0.259 compared to 0.205 in reference. In conclusion, the frequency of openings, both short and long, was enhanced by PKA. Furthermore, the occurrence of longer openings after filtering (τ_3), which might either represent bursts or actual openings (Ma et al., 1991), was enhanced, both in terms of absolute frequency, and relative to the occurrence of long openings (τ_2).

Kinetics of gating

We used ensemble averages of individual sweeps to analyze the kinetics of gating. Fig. 4 *A* shows a successive series of records obtained with a 5 s pulse to −10 mV (*top*) in the presence of PKA. A major difference in kinetics (compare Fig. 6 of Ma et al., 1991) was that a substantial frequency of openings remained at the end of the 5 s pulse. Panel B represents the average current over an ensemble of 384 sweeps. The continuous line is the best fit with Eq. 2 and yielded the parameters $I_{max} = 0.26$ (0.07) pA, $\tau_m = 130$ (6.8) ms and $\tau_h = 2.9$ (0.1) s. The general characteristics of channel gating were conserved before and after PKA, but the best fit parameters were different.

There seemed to be no change in the activation time constant in this particular average. However, the time resolution of the 5 s pulses is not the best to study the kinetics of activation. Therefore, activation kinetics, and the voltage-dependence of the effect, were studied with shorter records. For these purposes, 2 s pulses were used. Fig. 5 compares ensemble averages in reference at various voltages with averages after exposure to PKA. At

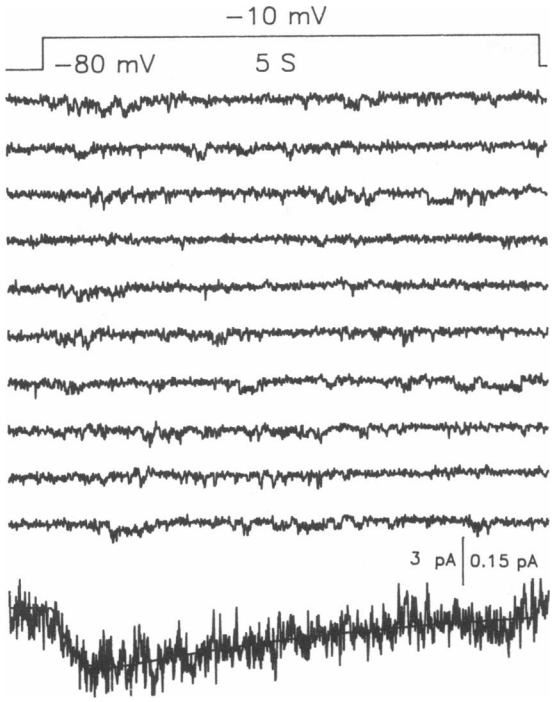

FIGURE 4 Ensemble averages of channel currents after exposure to PKA. Consecutive episodes, obtained at 7-s intervals, digitized at 200 Hz, starting 5 min after addition of 0.5 μM C-subunit of PKA. Bottom record: ensemble average of 402 episodes obtained in four bilayer experiments. The solid line was generated with Eq. 2 fitted to the average. Best fit parameters are: I_{max} = 0.26 (0.07) pA, τ_m = 132 (7) ms, τ_h = 2.9 (0.1) s.

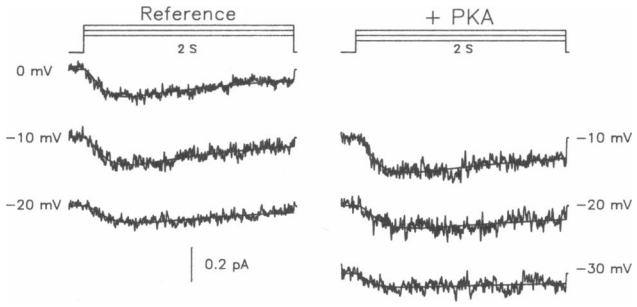

FIGURE 5 Effect of PKA on the voltage dependence of the ensemble-averaged current. Ensemble averages at the potentials indicated were generated from many episodes obtained with a 2-s depolarizing pulse. Blank sweeps were excluded from the averages. The number of episodes and number of experiments used are: at 0 mV (241, 5), at −10 mV (312, 12), at −20 mV (390, 11) for bilayers in reference conditions; −10 mV (320, 4), −20 mV (213, 9), −30 mV (225, 10) for channels treated with PKA. Seven of the nine bilayers at −20 mV and three of the 12 bilayers at −10 mV were studied both in reference and after PKA. Smooth lines represent the best fit with Eq. 2. Parameters are given in Table 1.

all voltages the inactivation was lower in PKA, and less complete after 2 s than in the reference condition.

The records in Fig. 5 were fitted with Eq. 2 and the best fit parameters are listed in Table 1. The fits listed in the table were obtained on collective averages that included records from between five and 12 different bilayers. In the collective fits, the activation time constant decreased by 8 ms at −10 mV, which is not significant, but was consistent with the left-shift of the activation curve. The only highly significant change in macroscopic gating caused by PKA was the increase in inactivation time constant, observed at both −10 and −20 mV. Because there is no evidence of voltage-dependence of τ_h, the results at both voltages could be pooled, giving an average value of 1.34 s for reference condition, and 2.50 s in PKA. This value agrees reasonably well with the more reliable estimate of the inactivation time constant in PKA (τ_h = 2.9 s) determined with pulses of longer duration (Fig. 4).

Deactivation

Fig. 6 shows records obtained at a high sampling rate (4 points/ms) and lower overall gain (100 mV/pA), conditions used in the previous paper to characterize the rapid closure after repolarization. A reduced pulse duration (400 ms) allowed us to observe deactivation when $P_0(t, V)$ was at its maximum both in reference (*left side panel*) and PKA. In the single channel records, episodes having openings that lasted several ms after the end of the pulse became relatively frequent in PKA. The ensemble averages (*bottom*) clearly show the tail current, which was fitted by an exponential of time constant

TABLE 1 Voltage dependence and effects of phosphorylation of the skeletal muscle Ca^{2+} channel

Voltage		τ_m	τ_h	I_{max}	M_0/M
mV		ms	s	pA	
0	Control	101 ± 2.5	1.535 ± 0.039	0.223 ± 0.004	0.22 ± .02
	+PKA				
−10	Control	118 ± 4	1.24 ± 0.04	0.25 ± 0.006	0.26 ± .03
	+PKA	110 ± 3	2.48 ± 0.09	0.26 ± 0.005	0.19 ± .02
−20	Control	137 ± 5	1.44 ± 0.06	0.16 ± 0.005	0.30 ± .02
	+PKA	130 ± 6	2.52 ± 0.18	0.18 ± 0.006	0.23 ± .02
−30	Control				
	+PKA	117 ± 8	4.07 ± 0.49	0.096 ± 0.004	0.28 ± 0.03

Ensemble averaged currents at each membrane potentials were fitted according to: $I = I_{max}(1 - e^{-t/\tau_m})^3 e^{-t/\tau_h}$. Numbers given for τ_m, τ_h, and I_{max} are best fits ± errors of fit. M_0/M = number of blank episodes/total number of episodes (mean value ± standard error of the mean). The number of experiments for each condition was between five and 12. The difference of the mean values of M_0/M (reference and + PKA) was significant at all voltages ($P < 0.04$).

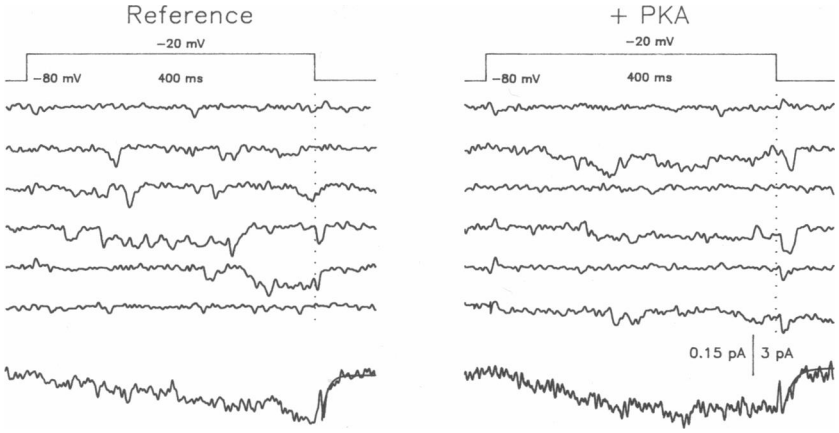

FIGURE 6 Effect of phosphorylation on channel deactivation. Consecutive episodes of 500 ms, recorded at a total gain of 100 mV/pA and digitized at a rate of 4 kHz in reference condition (*left*) and after PKA (*right*). Pulses of 400 ms to −20 mV. Records at bottom: ensemble averages of 110 episodes from four bilayers in reference (*left side*) and 80 episodes from three bilayers after PKA. The deactivation (*tail*) current was fitted by a single exponential decay to zero. The time constant was 8 (0.9) ms in reference and 12 (1.1) ms after PKA.

8 (0.9) ms in reference and 12 (1.1) ms in PKA. The difference of time constants was significant at a $P < 0.01$. The conclusion from these studies at higher time resolution is that, after PKA, the channels seemed to close more slowly upon repolarization.

Availability

To determine the effects of phosphorylation by PKA on channel availability, we analyzed the fraction M_0/M of null sweeps (Table 1, last column). The entries in the column are averages of separate determinations in five to 12 different bilayers. The numbers in parentheses are standard errors of the mean. PKA caused a significant decrease in M_0/M at both −10 and −20 mV. As was observed in reference, M_0/M was somewhat voltage dependent, decreasing at higher voltages. The difference in M_0/M between the highest and the lowest voltages was significant both in reference condition and after addition of PKA.

DISCUSSION

The major findings of the present work are that phosphorylation of the DHP-sensitive Ca channels from skeletal muscle by PKA results in: (*a*) an increased open probability; (*b*) a leftward shift in the voltage dependence of activation; (*c*) a decrease in the rate of inactivation; and (*d*) an increase in the availability of the channel to open.

Increase in channel open probability

The addition of PKA increased the open probability at all voltages studied. This overall effect was the result of several changes in gating. The $\bar{P}_0$ vs. V curve underwent a shift to the left by 7 mV and an increase in the value of $\bar{P}_{max}$, from 0.078 in reference to 0.12 after PKA. In the previous paper (Ma et al., 1991) it was shown that the open channel probability may be described as the product of two voltage- and time-dependent terms (m^3 and h) and P_{max}, the maximum open probability, a theoretical value that can only be reached in the situation in which the channel is fully activated by voltage and fully available. The factor P_{max} was pictured as the open probability of a voltage-independent "gate" that is in series with the voltage dependent gates, and that tends to be closed most of the time.

According to Eq. 14 of Ma et al. (1991):

$$\bar{P}_{max} = N P_{max} h(0) \beta \quad (3)$$

$$\beta \equiv [1 - \exp(-T/\tau_h)]/\tau_h/T, \quad (4)$$

where N is the number of channels in the bilayer (estimated at two or three), and β represents the effect of averaging over time a probability that is decaying due to inactivation. β depends on the pulse duration T and the inactivation time constant; using the values of τ_h found with pulses of 2 s, β changed from 0.5 in reference to 0.69 in PKA.

Because the main kinetic effect of PKA in the present study was a reduction in the rate at which the channels enter the inactivated state, a simple explanation of the

increase in availability is that the rate of inactivation was also decreased at the holding potential, which, in the presence of an unchanged or increased rate of recovery from inactivation, determined a lower steady state value of inactivation at the holding potential. If N channels are in the membrane:

$$M_0/M = [1 - h(0)]^N. \quad (5)$$

Because the fraction of nulls M_0/M changed from $\simeq 0.25$ to $\simeq 0.2$ (Table 1), $h(0)$ increased from $\simeq 0.43$ in reference to $\simeq 0.53$ in PKA. This implies, using Eq. 3, that P_{max} went from $\simeq 0.15$ to $\simeq 0.14$. From this approximate calculation, which depends on the estimate of N and assumes that this number does not change under PKA, the open probability of the voltage-dependent gate remained unchanged. The increase in $\bar{P}_0$, demonstrated by the curves of Fig. 2, was due mainly to a decrease in the inactivation rate at depolarized potentials (which increases β, Eq. 4) and an increase in availability at the holding potential ($h[0]$). The slight increase in the rate of activation in response to the depolarizing potential contributed only marginally to the increase in P_0.

Interestingly, open lifetimes of the channel were not changed by phosphorylation. The analysis of open times revealed mainly an increased proportion of bursting events (W_3, τ_3) (compare Fig. 3 *B* with Fig. 5 *D* of Ma et al., 1991).

Our results include the observation of a shift in the activation curve towards negative voltages of the phosphorylated channel. The shift, small but significant, probably is associated with the small increase in activation rate (which was not significant in the present measurements) and the decrease in deactivation rate. Such a shift has not been reported in skeletal muscle studies, but seems to be present in the results of Arreola et al., (1987) in which the threshold for activation of I_{Ca} is shifted to the left by some 5 mV. In ventricular myocytes, however, several recent observations suggest that cAMP and β adrenergic agonists shift the activation of Ca current to more negative voltages (reviewed by Pelzer et al., 1990).

Putative phosphorylation sites for PKA exist in the cytoplasmic loop between internal repeats II and III of the DHP receptor (Röhrkasten et al., 1988) and in the 300 residue-long, COOH-terminal, cytoplasmic segment (Tanabe et al., 1987). Phosphorylation of these cytoplasmic segments would add negative charges to the cytoplasmic side of the channel and conceivably could affect the transmembrane segments (S_4) believed to constitute the voltage sensitive portion of the DHP receptor (Tanabe et al., 1987). The simplest electrostatic consequence of the chemical modification should be a shift of the voltage dependent gating to more positive potentials. The fact that the opposite shift was observed, and that this shift was accompanied by other changes in kinetics, suggests that phosphorylation brings about changes in structure more complex than predicted by a simple addition of fixed negative charges.

Comparison with physiological studies in whole cells

The present results are not strictly comparable with previous work on skeletal muscle cells, due to the use of Bay K 8644 in our study. However, these results are consistent with most of the previous studies of the effects of phosphorylation and phosphorylation mediated interventions in muscle cells. Both β-adrenergic agonists and cAMP increase ^{45}Ca influx in cultured muscle cells (Schmid et al., 1985), as well as twitch tension (González-Serratos et al., 1981) and the rate of increase and peak value of I_{Ca} in skeletal muscle (Arreola et al. 1987). In addition, protein kinase A, applied intracellularly, increases peak I_{Ca} in cut fibers (García et al., 1990). The kinetic effects observed in the present study are also consistent in general with results on whole cells. Thus, the slight increase in the rate of activation is consistent with the reduction in time to peak of I_{Ca} that was observed in skeletal fibers treated with epinephrine or the catalytic subunit of PKA (Arreola et al., 1987). The slower inactivation observed here in the presence of PKA is consistent with the slower decay of Ca currents observed in the presence of PKA in rat muscle fibers (García et al., 1990). Only the observation of Arreola et al. (1987) of a faster decay of I_{Ca} in frog fibers in the presence of epinephrine is in disagreement with the present results. One explanation of this discrepancy is that the rates of decay measured by Arreola et al. (1987) were in part due to depletion of Ca^{2+} in the T tubules, and that this depletion occurred faster due to the increased I_{Ca} in the presence of epinephrine.

The effects of PKA on availability and inactivation rate observed here had the same direction as those of isoproterenol or epinephrine in cardiac Ca channels (Tsien et al., 1986; Trautwein and Pelzer, 1988; Ochi and Kawashima, 1990). The effects, however, were smaller in the present experiments with skeletal muscle channels. Considering that our experiments required Bay K 8644, an agent that increases significantly the slowing effect of isoproterenol on inactivation in cardiac cells (Tsien et al., 1986), the difference between the effects of phosphorylation on skeletal and cardiac channels may be even greater.

Comparison with biochemical studies of phosphorylation

The results add insight into the physiological significance of previous biochemical studies of phosphorylation of the channels by cAMP-dependent protein kinase, both in vitro and in vivo. Both the α_1 and β subunits serve as substrates in vitro for the cAMP-dependent protein kinase (Curtis and Catterall, 1986; Flockerzi et al., 1986; Nastainczyk et al., 1987; O'Callahan and Hosey, 1988; Jahn et al., 1988; Nunoki et al., 1989). The phosphorylation studies most relevant to the results presented here are those in which phosphorylation of the channels was performed directly in the T-tubule membranes (O'Callahan and Hosey, 1988; O'Callahan et al., 1988) rather than with purified channels. The conditions of the previous phosphorylation studies with T-tubule membranes were very similar to those used here and result in rapid and stiochiometric phosphorylation of the α_1 subunit, with a five-fold lower phosphorylation of the β subunit (Chang et al., 1990). The α_1 subunit undergoes rapid phosphorylation in intact skeletal muscle cells in response to stimulation of the cells with isoproterenol (Lai et al., 1990; Mundiña-Weilenmann et al., 1991). No phosphorylation of the β subunit was observed (Mundiña-Weilenmann et al., 1991).

The present results and the biochemical studies taken together suggest that regulation of skeletal muscle Ca channels by cAMP-dependent events occurs in intact muscle via phosphorylation of the α_1 subunit, and that several molecular transitions are affected, the consequence being an increased readiness of the channels to open in response to depolarizations.

It would be of interest to determine whether and how phosphorylation of the DHP receptors affects their role as voltage sensors of excitation-contraction coupling (Ríos and Brum, 1987; Tanabe et al., 1988). β-adrenergic agonists promote EC coupling (Brum et al. 1990), as does GTP γS (Di Virgilio et al., 1986; García et al., 1990). Additionally, recent observations on the effect of chimeric DHP receptors expressed in myotubes of myodysgenic mice showed that a cytoplasmic region of the skeletal muscle α_1 subunit, joining internal repeats II and III, is important in imparting the unique features that allow the DHP receptor to perform the voltage sensing function in skeletal-type excitation-contraction coupling (Tanabe et al., 1990). Because this region contains the preferred site of phosphorylation by the cAMP-dependent protein kinase in vitro (serine-687, Röhrkasten et al., 1988) it constitutes a prime candidate for the site at which phosphorylation could have E-C coupling effects. More needs to be done in order to establish if phosphorylation has a role in the modulation of E-C coupling. The present results, however, suggest that two specific effects should be sought, a leftward shift of the voltage dependent activation of E-C coupling, and a reduction in the rates of voltage-dependent inactivation.

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